REMARKS

These remarks are in response to the Final Office Action mailed July 7, 2006. Claims 1, 3 and 6 have been amended, and claim 2 has been canceled. Support for the recitation of "wherein the medium is not previously elaborated with cells derived from metanephric mesenchyme and does not contain detectable levels of pleiotrophin" can be found at paragraphs [0047] and [0048] of the application as published (US Patent Application Pub. No. 2005/0074875), and throughout the specification as filed. Applicants reserve the right to prosecute the canceled subject matter in any divisional, continuation, continuation-in-part or other application. No new matter is believed to have been introduced.

I. IN THE SPECIFICATION

The specification has been amended to reflect the priority claim, as required by 37 CFR 1.78. The Examiner states that the disclosure of the prior-filed applications, Application No. 09/956,651 and 09/595,195, fail to provide adequate support or enablement in the manner provided by the first paragraph of 35 U.S.C. 112, for claims 3-7 of this application. However, because there is sufficient support in the prior-filed applications for claims 1 and 2, the priority claim made herein is proper.

II. OBJECTIONS TO THE CLAIM

Claim 1 is objected to due to the incorrect spelling of "utereric". Applicants have amended the claim to correct the spelling error. Accordingly, the objection may be properly withdrawn.

III. REJECTION UNDER 35 U.S.C. §112, FIRST PARAGRAPH

Written Description

Claims 3-7 stand rejected under 35 U.S.C. §112, first paragraph as allegedly failing to comply with the written description requirement. In particular, the Examiner alleges that the claims contain subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the

claimed invention. Specifically, the Examiner alleges there is no evidence that the inventors were in possession of isolated active fragments of pleiotrophin and/or heregulin at the time of filing. Applicants respectfully traverse this rejection.

Applicants submit that active fragments of pleitrophin and heregulin are known in the art, as demonstrated in Zhang et al (1999). "A dominant-negative pleiotrophin mutant introduced by homologous recombination leads to germ-cell apoptosis in male mice," PNAS, 96(12):6734-6738, and Barbacci et al (1995). "The Structural Basis for the Specificity of Epidermal Growth Factor and Heregulin Binding," J Bio Chem, 270(16):9585-9589, attached hereto. The Examiner asserts that a lack of adequate written description issue arises if the knowledge and level of skill in the art would not permit one skilled in the art to immediately envisage the product claimed from the disclosed process. Applicants respectfully bring to the attention of the Examiner that the invention claimed herein is not to a pleiotrophin or heregulin factor, but rather to methods of cultivating cells. Thus, the requirement of evidence of possession of active fragments of pleiotrophin and heregulin is not proper because a patent need not teach, and preferably omits, what is well known in the art. Accordingly, withdrawal of this rejection is respectfully requested.

Further, the Examiner asserts that there is insufficient written description provided in the disclosure to adequately describe the precise action GDNF and/or FGF1 have on the branching morphogenesis of ureteric bud cells, and that there is no evidence that the inventors were in possession of functional equivalents of homologues of GDNF and/or FGF1. Applicants respectfully traverse.

As stated above, the invention claimed herein is not to a product, but rather to methods. Thus, the requirement of evidence of possession of functional equivalents of homologues of GDNF and/or FGF1 is not proper. Nevertheless, Applicants provide herewith APPENDIX A (Hsu et al. "Differential control of murine aldose reductase and fibroblast growth factor (FGF)-regulated-1 gene expression in NIG 3T3 cells by FGF-1 treatment and hypersomotic stress," Biochem. J. 328:593-598) which evidences the biologic function of FGF1 at the time of filing. Accordingly, withdrawal of this rejection is proper and respectfully requested.

Claims 1-7 stand rejected under 35 U.S.C. §112, first paragraph as allegedly failing to comply with the written description requirement. In particular, the Examiner alleges that while being enabling for a method of inducing ureteric bud cells to undergo branching morphogenesis in culture comprising culturing ureteric bud cells in either BSN-CM or pleiotrophin, it allegedly does not reasonably provide enablement for inducing UB cells to undergo branching morphogenesis in culture comprising only heregulin. As demonstrated in Sakurai et al ("Heregulin Induces Glial Cell Line-derived Neurotrophic Growth Factor-independent, Non-branching Growth and Differentiation of Ureteric Bud Epithelia," J Bio Chem, 280(51):42181-42187), attached hereto as APPENDIX B, heregulin is used alone to induce UB cells to proliferate in culture, (see, e.g., the second column of page 42183). This is consistent with paragraphs [0055] and [0140] of the specification. Accordingly, Applicants submit that the specification does indeed enable a person skilled in the art to make or use the invention commensurate in scope with the claims herein as evidenced by the Sakurai article. Applicants respectfully request withdrawal of this rejection.

IV. REJECTION UNDER 35 U.S.C. §112, SECOND PARAGRAPH

Claims 1-7 stand rejected under 35 U.S.C. §112, second paragraph as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Specifically, the Examiner alleges that is unclear what 'conditions' induce the UB to undergo branching morphogenesis, as recited in claim 1. Applicants have amended claim 1 to clarify the conditions that induce the UB to undergo branching morphogenesis. Accordingly, withdrawal of this rejection is proper and respectfully requested.

Further, the Examiner asserts that the step of culturing the isolated ureteric bud tissue under 'sufficient conditions' is unclear. Applicants have amended claim 3 to delete reference to 'sufficient conditions' in order to clarify the claimed method. Accordingly, withdrawal of this rejection is proper and respectfully requested.

V. REJECTION UNDER 35 U.S.C. §103

Claims 1, 3, 6 and 7 stand rejected under 35 U.S.C. §103, as allegedly unpatentable over Sakurai et al. (PNAS, 1997), in view of "Basic Techniques for Mammalian Cell Tissue Culture" (Current Protocols in Cell Biology, 1998), Naughton et al. (US 2003/0007954), and "Overview of Extracellular Matrix" (Current Protocols in Cell Biology, 1998). Applicants respectfully traverse this rejection.

Applicants submit that Sakurai et al. fails to teach or suggest the combination of GDNF and BSN-CM as recited in amended claim 1. Applicants direct the Examiner to Figure 5 (page 6283 of Sakurai et al.) in which the cited reference suggests only the use of each growth factor alone or the growth factor in combination (i.e., GF-mix) without BSN-CM. In contrast, one of skill in the art upon reading Sakurai et al., would not have been motivated to use GDNF as this growth factor alone had only minimal activity compared to IGF and FGF (see, e.g., FIG. 5 of Sakurai et al.). Thus, there would have been no motivation to combine GDNF with BSN-CM as currently recited in Applicants' claims.

Applicants respectfully submit that the Examiner is using hindsight to arrive at the claimed invention. For example, the Office Action alleges that it would have been obvious to arrive at Applicants' invention based upon the reading of Sakurai et al. because it would have been " well within the purview of one of ordinary skill in the art" as part of routine animal cell tissue culture methods, to divide and resuspend subpopulations of the UB cells (claims 1 and 3). Applicants submit that with such global knowledge imputed to one of skill in the art it would be obvious in hindsight to arrive at almost any invention with the mere motivation of continually subculture the growing of cell tissue culture of a species because the genus cell tissue culture techniques are well known in the art.

Applicants submit that the there is no motivation to combine the references to arrive at Applicants' invention. The motivation to arrive at Applicants' invention can only be found in hindsight reconstruction particularly where, as the case is here, the teachings clearly indicate that GDNF is a poor morphogenic factor. For at least the foregoing reasons, Applicants submit that Applicants invention is non-obvious over the cited references. Accordingly, Applicants respectfully request withdrawal of the rejection.

Claims 1, 3, 4, 6 and 7 stand rejected under 35 U.S.C. §103, as allegedly unpatentable over Qiao et al., in view of "Basic Techniques for Mammalian Cell Tissue Culture" (Current Protocols in Cell Biology, 1998), Naughton et al. (US 2003/0007954), Naughton et al., and "Overview of Extracellular Matrix" (Current Protocols in Cell Biology, 1998). Applicants respectfully traverse this rejection.

Applicants note that the claimed methods are based, in part, on the discovery that morphogenic factors are secreted by metanephric mesenchyme cells. The Applicants have fractionated media conditioned by such cells and have identified at least some of the factors. In support, Applicants point to the following paragraphs set forth in the present specification:

> [0047] The invention demonstrates that UBs undergo branching tubulogenesis in the presence of a **conditioned medium elaborated by a cell line derived from the MM** also isolated from an E11.5 mouse (BSN cells). This suggests that other soluble factors present in BSN-CM are important for UB morphogenesis. These novel factors that are secreted by the MM are important for the development of the collecting system in artificial systems as well as in vivo. (emphasis added)
>
> [0049] The invention demonstrates that serial liquid column chromatographic fractionation of BSN-CM contain an active morphogenetic fraction comprising a single polypeptide (capable of inducing branching morphogenesis comparable to whole BSN-CM). **This polypeptide was identified as pleiotrophin** (FIG. 2). Immunoblot analysis of BSN-CM (FIG. 7A) as well as in situ hybridization data of developing kidney (Vanderwinden et al., 1992), demonstrate that the embryonic MM is a source of pleiotrophin. In addition to its ability to induce branching morphogenesis in the isolated UB, pleiotrophin also induced a UB cell line to form branching tubular structures with lumens, and is thus the only soluble factor so far identified with this capability (FIG. 6). Based on this in vitro data with the isolated UB as well as the UB cell line, the invention provides methods and compositions for use in vitro and in vivo to induce morphogenesis and tubular formation of tissues (e.g., kidney tissue). (emphasis added)
>
> [0050] The invention provides a novel factor, and combination of factors capable of inducing UB branching morphogenetic activity. In one aspect, the invention provides an **18 kDa heparin binding protein**, pleiotrophin, obtained from the BSN-CM. **This factor has not**

> **previously been shown to play a role in kidney morphogenesis.** (emphasis added)

Prior to this discovery, it was not known which factors were required for branching morphogenesis in kidney tissue. Not only does the cited reference of Qiao fail to identify such factors, it effectively teaches away from heparin binding proteins that are less than 100 kDa and promote branching morphogenesis. In support, Applicants point to page 7334, column 1, lines 8-11, which recite:

> "These preliminary data indicate that a heparin-binding protein or set of proteins greater than 100 kDa is one of the key morphogenetic activities in BSN-CM."

Clearly the cited reference fails to isolate or teach a heparin-binding protein that is less than 100 kDa in size and promotes branching morphogenesis. It is unclear how the claimed methods are obvious over Qiao in view of the absence of such information in the cited reference. The claims as amended recite methods in which cells are cultivated in a medium containing factor not previously identified as possessing branching morphogenetic properties.

To reach a non-hindsight driven conclusion as to whether a person having ordinary skill in the art at the time of the invention would have viewed the subject matter as a whole to have been obvious in view of multiple references, the Examiner must provide some rationale, articulation, or reasoned basis to explain why the conclusion of obviousness is correct. Applicants submit that such rationale, articulation, or reasoned basis is unlikely in view of the fact that none of the cited references teach or describe, individually or in combination, any connection between the presence of pleiotrophin in a medium and the promotion of branching morphogenesis.

In view of the above discussion and in light of the amendments to the claims, Applicants request withdrawal of the pending rejection.

No fee is believed to be due with respect to the filing of the present response. However, the Commission is authorized to charge any required fee, or credit any overpayment, to Deposit Account No. 02-4800.

Respectfully submitted,

BUCHANAN INGERSOLL& ROONEY LLP

Date: 10-10-06

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Appendix A

Biochemical Journal

Biochem. J. (1997) 328 (593–598) (Printed in Great Britain)

Differential control of murine aldose reductase and fibroblast growth factor (FGF)-regulated-1 gene expression in NIH 3T3 cells by FGF-1 treatment and hyperosmotic stress

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Abbreviations used: AR, aldose reductase; FGF, fibroblast growth factor; FR, FGF-1-regulated; GST, glutathione S-transferase; MAP, multiple antigen peptide; MVDP, mouse vas deferens protein.

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Aldose reductase (AR) is an NADPH-dependent aldo–keto reductase implicated in cellular osmoregulation and detoxification. Two distinct murine genes have been identified that are predicted to encode proteins with significant amino acid sequence identity with mouse AR: mouse vas deferens protein and fibroblast growth factor (FGF)-regulated-1 protein (FR-1). Here we report that the AR and FR-1 genes are differentially regulated in NIH 3T3 fibroblasts. FGF-1 stimulation of quiescent cells induces both AR and FR-1 mRNA levels, but the effect on FR-1 mRNA expression is significantly greater. FGF-1 treatment also increases FR-1 protein expression, as determined by Western-blot analysis using FR-1-specific polyclonal antiserum. Calf serum stimulation of quiescent cells increases AR mRNA expression but not FR-1 mRNA expression. Finally, when NIH 3T3 cells are grown in hypertonic medium, AR mRNA levels are significantly increased whereas FR-1 mRNA levels are only slightly up-regulated. These results indicate that the AR and FR-1 genes are differentially regulated in murine fibroblasts by two different growth-promoting agents and by hyperosmotic stress. Therefore these structurally related enzymes may have at least some distinct cellular functions; for example, although both AR and FR-1 activity may be important for the metabolic changes associated with cellular proliferation, AR may be the primary aldo–keto reductase involved in cellular osmoregulation.

INTRODUCTION

The aldo–keto oxidoreductases are a diverse family of monomeric enzymes that catalyse the NADPH-dependent reduction of various carbonyl substrates (reviewed in [1]). Aldose reductase (AR; EC 1.1.1.21), one of the most extensively characterized members of this family, can catalyse the NADPH-dependent conversion of glucose into sorbitol, the first step in the polyol pathway of carbohydrate metabolism (reviewed in [2]). In most circumstances, there is only a small amount of glucose flux through this pathway; however, when blood glucose levels are elevated (e.g. in diabetes mellitus) there is enhanced polyol pathway activity and, as a consequence, intracellular

sorbitol accumulation. This produces a hyperosmotic effect which is believed to be important for the development of several diabetes-associated complications (reviewed in [2–4]). Indeed, recent experiments using transgenic mice have provided direct evidence that AR-mediated polyol accumulation can promote diabetic cataracts [5,6] and neuropathy [7]. In addition to cellular osmoregulation, AR is likely to play an important role in several other processes, including the oxidoreduction of steroids within reproductive tissues [8] and the cellular catabolism of cytotoxic aldehydes [9,10].

Three distinct murine genes have been identified that are predicted to encode aldo–keto oxidoreductases with significant amino acid sequence identity with rat and human AR. In 1990, Pailhoux et al. [11] reported the isolation of a cDNA clone encoding a 316-amino acid polypeptide which they named mouse vas deferens protein (MVDP). This protein has approx. 70% amino acid sequence identity with rat and human AR. Subsequent studies have indicated that the MVDP gene is regulated by androgens [11–13] and is expressed primarily, but not exclusively, in the epithelium of the vas deferens [14,15]. It is at present not known whether MVDP is in fact a functional aldo–keto reductase. In 1994, our group reported the identification of a fibroblast growth factor (FGF)-1-inducible gene in murine NIH 3T3 cells predicted to encode a 316-amino acid aldo–keto reductase with approx. 82% sequence identity with MVDP and approx. 69% identity with rat and human AR [16]. This gene, named FGF-regulated (FR)-1, is expressed in a tissue-specific manner in the adult mouse, with highest mRNA levels detected in adrenal gland, heart, intestine, lung, ovary and testis [15,16]. Wilson et al. [17] have demonstrated recently that recombinant FR-1 exhibits NADPH-dependent reductase activity with D,L-glyceraldehyde as a substrate and that zopolrestat, a potent AR inhibitor, can also inhibit FR-1 activity. Furthermore these investigators were able to determine the crystal structure of the FR-1–NADPH–zopolrestat ternary complex at 1.7 Å resolution. The third and most recently identified murine aldo–keto reductase gene reported to date encodes a 316-amino acid protein with approx. 97% sequence identity with rat AR, approx. 85% identity with human AR and approx. 69% identity with mouse MVDP or FR-1 [18,19]. This gene is likely to be the murine homologue of rat and human AR and is expressed primarily in the kidney, skeletal muscle and testis of adult mice. Recombinant mouse AR protein is enzymically active, demonstrating reductase activity for various aldo sugars but not for steroids [18].

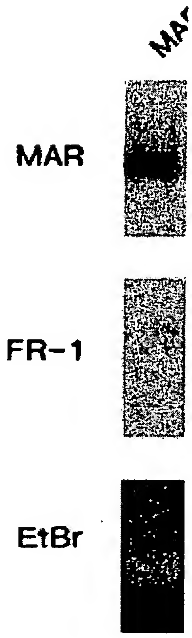


Figure 1 Hybridi of the murine AF probes

Here, we report that AR and FR-1 gene expression in murine NIH 3T3 fibroblasts is differentially regulated by FGF-1 treatment, calf serum treatment and hyperosmotic stress. We also describe the generation of anti-FR-1 polyclonal antiserum and show that FGF-1 stimulation of quiescent fibroblasts promotes FR-1 protein accumulation.

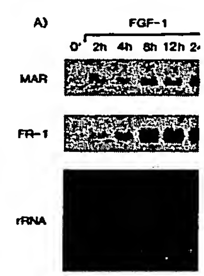


Figure 2 Effect o FGF-1 or calf se FR-1 mRNA leve fibroblasts

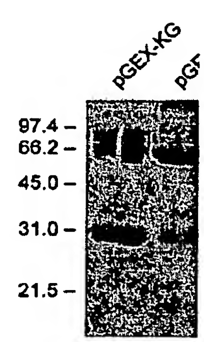
MATERIALS AND METHODS

Cell culture

Murine NIH 3T3 cells (American Type Culture Collection) were maintained, expanded and serum-starved as described [21]. For the growth-stimulation experiments, serum-starved cells were treated for various lengths of time with either 10 ng/ml recombinant human FGF-1 (gift from W. Burgess, Holland Laboratory) in combination with 5 units/ml heparin (Upjohn) or with 10% calf serum (Hyclone Laboratories). Cells were harvested by trypsin/EDTA treatment. For the hyperosmotic stress experiments, cells were first grown to approx. 80% confluency in normal culture medium. The medium was then replaced with either fresh isotonic medium (110 mM NaCl, 25 mM glucose) or fresh medium supplemented with 190 mM NaCl, 190 mM raffinose, 190 mM sorbitol, 275 mM glucose, 275 mM 3-*O*-methylglucose or 275 mM mannitol. The cells were then cultured for various lengths of time in either the isotonic or hypertonic medium and then harvested as described above.

In vitro transcription assays

The mouse AR plasmid (pDIRECT/ALR2; gift from J. Mark Petrash, Washington University School of Medicine) and the mouse FR-1 plasmid (pGEM3Zf+/FR-1 [16]) were each linearized by *Hin*dIII digestion. Sense strand transcripts were then synthesized *in vitro* using T7 RNA polymerase and the Ribomax large-scale RNA production system (Promega). RNA concentrations were determined by UV light absorbance at 260 nm.



RNA isolation and Northern-blot hybridization analysis

Total cellular RNA was isolated using RNA Stat-60 (Tel-Test Inc.) according to the manufacturer's instructions. RNA concentrations were calculated by measuring UV light absorbance at 260 nm. Each RNA sample (10 μg) was denatured and subjected to electrophoresis in 1.2% agarose gels containing 2.2 M formaldehyde. The gels were stained with ethidium bromide to verify that each lane contained similar amounts of undegraded rRNA. RNA was electroblotted on to Zetabind nylon membranes (Cuno Inc.) and cross-linked by UV irradiation using a Stratalinker (Stratagene). Northern-blot hybridization analysis was performed as described [22] using either the 1.1 kb *Hin*dIII–*Nco*I fragment of pDIRECT/ALR2 or the 1.3 kb *Eco*RI fragment of pGEM3Zf+/FR-1 (see above for sources of plasmids). The probes were radiolabelled with [α-^{32}P] dCTP (3000 Ci/mmol; Amersham) using a random-primed DNA-labelling kit (Boehringer-Mannheim) according to the manufacturer's instructions. The blots were air-dried and autoradiography was performed using Kodak X-Omat AR film. Hybridization signal intensities were quantified by densitometry using the BioImage whole-band analyzer (Millipore).

FR-1 expression and purification

Recombinant FR-1 was obtained by expression of the cDNA in *Escherichia coli* as a glutathione S-transferase (GST) fusion protein. The expression plasmid pGEX-KG/FR-1 was constructed by ligation of an *Nco*I–*Sal*I restriction fragment of pGEM3Zf+/FR-1 into the bacterial expression vector pGEX-KG (gift from R. Friesel, Holland Laboratory [23]). Overnight cultures of *Escherichia coli* HB101 (Gibco/BRL) transformed with either pGEX-KG or the pGEX-KG/FR-1 plasmid were diluted 1:10 in Luria broth and grown at 37 °C for 1 h. Isopropyl β-thiogalactoside (Gibco/BRL) was then added to a final concentration of 0.2 mM, and 3–4 h later cells were pelleted and resuspended in PBS supplemented with 1 mM PMSF (Sigma) and 1 mg/ml lysozyme (Sigma). Cells were lysed by incubation at 37 °C for 5 min, Triton X-100 (Sigma) was added to a 1% (v/v) final concentration, and mild sonication was used to reduce viscosity. The lysates were then subjected to two cycles of centrifugation at 10000 ***g*** at 4 °C for 5 min. The supernatants were then incubated with hydrated glutathione–Sepharose 4B (Pharmacia) at 4 °C for 30 min with end-over-end mixing. The beads were collected by brief centrifugation at 500 ***g*** and washed 5 times with PBS containing 1% (v/v) Triton X-100. For protease cleavage of the GST–FR-1 fusion protein, the beads were washed once with 50 mM Tris/HCl, pH 8.0/150 mM NaCl/ 2.5 mM $CaCl_2$ and then incubated in 0.5 ml of this buffer containing 10 units/ml bovine thrombin (Armour) and 30 mM 2-mercaptoethanol (Sigma) at room temperature for 30 min with end-over-end mixing. The beads were collected by brief centrifugation and the supernatant (containing FR-1) was stored at -80 °C. The FR-1 concentration was determined using the BCA protein assay reagent (Pierce Chemical Co.). All samples were analysed by SDS/PAGE followed by staining with Coomassie Blue.

Generation and purification of anti-FR-1 serum

An FR-1 peptide was synthesized on an octa-branched core matrix using the multiple antigen peptide (MAP) approach described by Tam [24]. Briefly, the peptide LLPETVNMEEYPYDAEY (residues 300–316 of FR-1 [16]) was synthesized on Fmoc (fluoren-9-ylmethoxycarbonyl)-MAP resin (eight-branch; Applied Biosystems), cleaved from the resin with trifluoroacetate, and then purified by reverse-phase HPLC. A New Zealand White rabbit was first injected with approx. 0.5 mg of the antigen in complete Freund's adjuvant (Calbiochem) and then boosted 4 times with approx. 0.3 mg of the antigen in incomplete adjuvant (Calbiochem). For affinity purification of the antiserum, recombinant FR-1 protein was immobilized on to a methanol-pretreated ProBlot membrane (Applied Biosystems) by immersing the membrane in 10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl, 0.05% (v/v) Triton X-100 and 2.5 μg/ml FR-1 at room temperature for 4 h. The membrane was washed with the above solution (minus protein) for 10 min to remove unbound FR-1 and then blocked by immersion in 10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl and 4% (w/v) BSA (Boehringer-Mannheim) at room temperature for 2 h. An aliquot of antiserum was then diluted 2-fold using 10 mM Tris/HCl, pH 7.4, containing 150 mM NaCl and 0.05% (v/v) Triton X-100 and incubated with the membrane at 4 °C for 18 h on a rocking platform. The membrane was then washed twice (5 min each) with the above solution, and antiserum eluted by immersion in 0.2 M glycine, pH 2.8, with vigorous agitation for 15 min. The antiserum was then neutralized using 1 M Tris/HCl, pH 8.0, and stored at -20 °C.

Western-blot analysis

Recombinant FR-1 protein was purified as described above and recombinant rat AR protein was a gift from D. Carper (National Eye Institute, Bethesda, MD, U.S.A.). Cellular lysates were prepared as described [21] and the amount of protein in each clarified lysate was determined using the BCA protein assay reagent. Equivalent amounts of each protein sample (0.2 μg of each

Figure 3 Expres purification of re FR-1

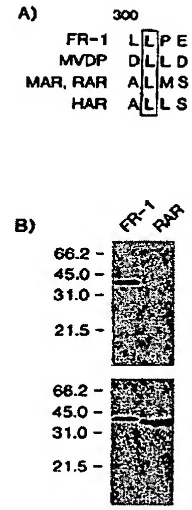


Figure 4 Reactiv of the anti-FR-1 antiserum

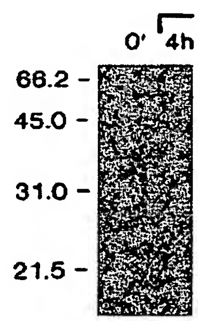


Figure 5 Effect o on FR-1 protein fibroblasts

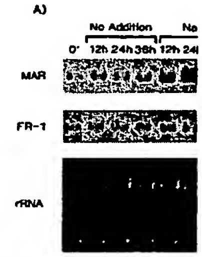


Figure 6 Effect o stress on AR an levels in NIH 3T

recombinant protein or 50 μg of NIH 3T3 cell protein) were mixed with 2×sample buffer [125 mM Tris/HCl, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol], heated at 95 °C for 4 min, and subjected to SDS/PAGE using either a 15% (recombinant protein) or 12% (cellular lysates) polyacrylamide/SDS slab gel. Low-molecular-mass protein standards (Bio-Rad Laboratories) were also included on the gels. Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell) by electroblotting. The membranes were stained with Ponceau S (Sigma) to verify that equivalent amounts of protein were present in each gel lane and then incubated in TBST/milk [50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 0.5% (v/v) Tween 20, 5% (w/v) non-fat dry milk] at 4 °C for 18 h. The membranes were then incubated in TBST/milk containing either a 1:1000 dilution of anti-FR-1 serum or a 1:250 dilution of anti-AR serum (also a gift from D. Carper) at room temperature for 2 h, washed twice with TBST/milk, and then incubated in TBST/milk containing a 1:20000 dilution of horseradish peroxidase-conjugated goat anti-rabbit IgG secondary antibody (Bio-Rad Laboratories) at room temperature for 1 h. Bound secondary antibody was detected using an enhanced chemiluminescence kit (Amersham). The blots were air-dried and autoradiography was performed using Kodak X-Omat AR film.

RESULTS AND DISCUSSION

Differential regulation of AR and FR-1 mRNA expression by cell-growth promoters

In a previous report, we demonstrated by Northern-blot hybridization analysis that FGF-1 stimulation of quiescent NIH 3T3 fibroblasts increased FR-1 mRNA levels and that this cellular response required *de novo* RNA and protein synthesis [16]. FGF-2 treatment also induced FR-1 mRNA expression but treatment with other polypeptide mitogens (e.g. platelet-derived growth factor) or with calf serum (which contains numerous growth-promoting factors) had no significant effect. In order to determine whether FGF-1 stimulation of NIH 3T3 cell growth also induced AR mRNA expression, we obtained a murine AR cDNA clone to use as a Northern-blot hybridization probe. The murine AR and FR-1 cDNAs have approx. 65% nucleotide sequence identity; thus one would predict that they should only recognize their corresponding mRNAs when used as probes in high-stringency hybridization experiments. In our initial experiments, we confirmed that this was indeed the case by Northern-blot analysis using *in vitro* transcribed AR and FR-1 mRNA (Figure 1).

We then examined AR and FR-1 mRNA levels in FGF-1- or calf serum-treated NIH 3T3 cells by Northern-blot hybridization analysis. Serum-starved cells were either left untreated or treated with FGF-1 or calf serum for different lengths of time and then harvested. RNA was isolated and equivalent amounts analysed by Northern-blot hybridization. The AR and FR-1 transcripts were each approx. 1.4 kb in size (consistent with previous reports [16,18]) and were detected in both quiescent and growth-promoter-stimulated cells. FGF-1 treatment increased both AR and FR-1 mRNA expression levels, although FR-1 mRNA induction was more pronounced (Figure 2A). Increased AR and FR-1 mRNA levels were first apparent at 4 h, with maximal AR mRNA levels (approx. 5.8-fold induction) and FR-1 mRNA levels (approx. 13.8-fold induction) detected at 24 h after FGF-1 addition. Calf serum treatment also increased AR mRNA expression, with an approx. 2.9-fold level of induction detected at 8 h after serum addition; however, FR-1 mRNA levels were only slightly increased (Figure 2B). The observed changes in AR and FR-1 mRNA levels after FGF-1 or calf serum addition do not appear to reflect unequal loading of the RNA samples since similar amounts of 28 S and 18 S rRNA were present in each gel lane. We attempted to confirm that equivalent amounts of RNA were loaded in each gel lane by reprobing the Northern blots with a β-actin cDNA clone, but we found that β-actin mRNA expression was up-regulated after mitogenic stimulation of quiescent cells (results not shown). This result is consistent with previous reports demonstrating that β-actin is a growth-factor-regulated immediate-early response gene (reviewed in [25]).

The above results demonstrate that (i) FGF-1-stimulated cells express elevated levels of both the AR and FR-1 mRNAs, but these two transcripts have different maximal induction levels, (ii) calf serum-stimulated cells express significantly elevated levels of AR mRNA but not FR-1 mRNA, and (iii) although both FGF-1 and calf serum treatment of quiescent cells can induce AR mRNA levels, the effect of each growth-promoting agent is distinct. The results reported here on the effect of FGF-1 or calf serum on FR-1 mRNA expression in NIH 3T3 fibroblasts are consistent with our previous study [16]. FGF-1 and the related mitogen FGF-2, which can also induce FR-1 [16] and AR (results not shown) mRNA levels in these cells, have been shown to regulate AR gene expression in two other experimental systems. Peek et al. [26] reported that increased AR mRNA expression was associated with FGF-2-mediated rat lens epithelial-cell terminal differentiation, and Jacquin-Becker and Labourdette [27] reported that AR gene expression was up-regulated after FGF-1 or FGF-2 treatment of rat astrocytes. Taken together with our results, it

appears that FGF-1- or FGF-2-stimulated cellular proliferation as well as differentiation can be accompanied by elevated AR and/or FR-1 gene expression. The physiological role of increased AR or FR-1 expression in these contexts is at present not clear, but these aldo–keto reductases may be important for the profound changes in cellular metabolism that are associated with these complex processes.

Regulation of FR-1 protein expression by FGF-1 treatment

We next investigated whether the increased expression of FR-1 mRNA in FGF-1-stimulated NIH 3T3 cells resulted in a detectable increase in FR-1 protein levels. To address this issue, we had to generate an immunological reagent that would recognize FR-1 but not AR. This was accomplished using the following approach. First, we expressed FR-1 as a GST fusion protein in bacteria and then used recombinant protein for both affinity purification of crude antiserum and for testing antiserum specificity. The full-length FR-1 cDNA was subcloned into the pGEX-KG expression vector and the parental and recombinant plasmids individually transformed into bacterial cells. Cultures were treated with isopropyl β-thiogalactoside, and GST itself as well as the GST–FR-1 fusion protein were purified from bacterial lysates by affinity chromatography on glutathione–Sepharose. An aliquot of the immobilized GST–FR-1 fusion protein was then cleaved with thrombin to obtain recombinant FR-1. The purified GST, GST–FR-1 and FR-1 proteins were the appropriate sizes (approx. 26, 62 and 36 kDa respectively) as determined by SDS/PAGE and Coomassie Blue staining (Figure 3). Second, we immunized rabbits with an octa-branched synthetic MAP corresponding to the C-terminal 17 amino acid residues of the predicted FR-1 protein. This peptide was chosen because murine AR, MVDP and FR-1 have a relatively low degree of sequence identity in their C-terminal regions (Figure 4A). Western-blot analysis using affinity-purified anti-FR-1 serum indicated that it recognized FR-1 but not rat AR, which has the same C-terminal sequence as mouse AR (Figure 4B). In contrast, polyclonal antiserum generated against recombinant rat AR protein recognized both FR-1 and rat AR. This latter finding indicates that previous Western blot, ELISA or immunohistochemical studies that utilized antiserum generated using purified AR protein as the antigen could be detecting AR as well as AR-related proteins that may be expressed in the cells or tissues under investigation.

Western-blot analysis was then performed to investigate whether FGF-1 treatment of NIH 3T3 cells resulted in elevated FR-1 expression. Serum-starved cells were either left untreated or treated with FGF-1 for different lengths of time. Cells were harvested and equivalent amounts of protein were analysed by SDS/PAGE and Western blotting using the anti-FR-1 serum. A major immunoreactive protein of approx. 36 kDa, a size consistent with the predicted molecular mass of FR-1 [16], was detected in FGF-1-treated cells (Figure 5). FGF-1 stimulation increased FR-1 expression, and the temporal expression kinetics of FR-1 accumulation were somewhat delayed compared with that of FR-1 mRNA accumulation. These results demonstrate that FR-1 protein induction is associated with FGF-1-mediated cell cycle progression.

Differential regulation of AR and FR-1 mRNA expression by hyperosmotic stress

Previous reports have demonstrated that AR gene expression is induced when various cell types (e.g. rat astrocytes [27], human renal proximal tubule cells [28], human retinal pigment epithelial cells [29], dog lens epithelial cells [30], rabbit renal medullary cells [31], Chinese hamster ovary cells [32]) are cultured under conditions of hypertonicity. Indeed, an osmotic response element (also referred to as a tonicity-responsive element) has been identified in the promoter regions of the human [33], rabbit [34] and mouse [35] AR genes. Therefore we determined whether AR and/or FR-1 gene expression in NIH 3T3 fibroblasts was regulated by hyperosmotic stress. Cells were grown to subconfluent density in normal, isotonic culture medium and then the medium was replaced with either fresh isotonic medium or hypertonic NaCl-supplemented medium. Cells were cultured under these conditions for different lengths of time and then harvested. RNA was isolated and equivalent amounts analysed by Northern-blot hybridization. Hyperosmotic stress significantly increased AR mRNA levels but had only a small stimulatory effect on FR-1 mRNA expression (Figure 6A). AR mRNA levels gradually increased after exposure to hypertonic medium, with approx. 3.0-, 7.9- and 14.8-fold levels of induction apparent at 12, 24 and 36 h respectively.

We next investigated whether AR or FR-1 gene induction occurred in response to hyperosmolarity produced by other compounds, specifically raffinose, sorbitol, glucose, 3-*O*-methylglucose (a non-metabolizable glucose analogue) or mannitol. In this case, cells were harvested after growth in hypertonic medium for 24 h, RNA was isolated and Northern-blot hybridization analysis performed. AR mRNA levels, but not FR-1 mRNA levels, were significantly elevated by hypertonic stress induced by all of these compounds, with levels of induction similar to that noted in NaCl-

supplemented medium (Figure 6B).

The changes in AR mRNA levels observed in the above experiments do not appear to reflect unequal loading of the RNA samples since similar amounts of 28 S and 18 S rRNA were present in each gel lane. We attempted to confirm that equivalent amounts of RNA were present in each gel lane by reprobing the Northern blots with a β-actin cDNA clone, but we found that β-actin mRNA expression was down-regulated in hypertonic medium (results not shown). This finding indicates that decreased expression of cytoskeletal proteins such as β-actin may be required for the changes in cell shape associated with osmotic stress. Taken together, the above results demonstrate that the mouse AR and FR-1 genes are differentially regulated by hyperosmolarity and that AR, but not FR-1, is likely to be involved in cellular osmoregulation.

In summary, the results described above indicate that, although the murine AR and FR-1 genes are co-expressed in a fibroblast cell line, they are differentially regulated by growth-promoting agents as well as by hypertonic stress. Specifically, FGF-1 treatment of quiescent cells induces both AR and FR-1 mRNA levels (albeit to different degrees), whereas serum treatment of quiescent cells or cell growth in hypertonic medium primarily results in up-regulation of AR mRNA expression. It is therefore likely that these two structurally related NADPH-dependent aldo–keto reductases have unique enzymic functions in mammalian cells.

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Appendix B

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Heregulin Induces Glial Cell Line-derived Neurotrophic Growth Factor-independent, Non-branching Growth and Differentiation of Ureteric Bud Epithelia*

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We have purified a protein present in a conditioned medium derived from the metanephric mesenchyme that supports non-branching growth and epithelial differentiation of the isolated ureteric bud (UB) independent of glial cell line-derived neurotrophic growth factor (GDNF). By sequential liquid chromatography, together with protein microsequencing, the protein was identified as heregulin (HRG) α. The addition of recombinant HRG to the isolated UB grown in three-dimensional culture confirmed the proliferative activity of HRG. In branching UBs induced by whole metanephric mesenchyme cell-conditioned medium, proliferating cells were localized at ampullae, where a binding receptor for GDNF, GFRα1, was found. In HRG-induced UBs, however, the expression of GFRα1 was down-regulated, and proliferating cells were distributed throughout the structure. Electron microscopic examination of the HRG-induced UB revealed the presence of structurally mature and polarized epithelial cells reminiscent of the epithelial cells found in the stalk portion of the branching UB. cDNA array analysis further revealed that genes ontologically classified as developmental were down-regulated by HRG, whereas those involved in transport were up-regulated. For example, the mRNA for the GDNF receptors, GFRα1 and ret9, was down-regulated, whereas the mRNA for collecting duct transporters, such as urea transporter2, aquaporin3, and sodium-hydrogen exchanger2 was up-regulated in HRG-treated UBs compared with UBs grown in the presence of branch-promoting factors. Moreover, HRG promoted growth of UBs cultured in the absence of GDNF. Taken together, the data suggest that HRG supports UB epithelial cell differentiation and non-GDNF-dependent growth, raising the possibility that this kind of activity plays a role in the growth and differentiation of the stalk portion of the branching epithelial UB.

Branching morphogenesis is essential for the development of many epithelial organs including the lung, salivary gland, mammary gland, and kidney. In the kidney, growth factors such as glial cell line-derived neurotrophic growth factor (GDNF),[5] pleiotrophin (PTN), fibroblast growth factor (FGF)s, and transforming growth factor-β superfamily have been implicated in this process. However, beyond cell proliferation and survival, how the actions of these growth factors translate into forming a branched tubular or ductal structure is not well understood.

To form a branching structure, it appears necessary to have clear tip and stalk regions. In fact, it has recently been shown that there are at least two distinct cell types in the developing ureteric bud, ret-expressing tip cells and ret-negative stalk cells (1). These cells clearly have different fates, tip cells are fated to remain within the tip and make new branches or to move toward the stalk region to become stalk cells, whereas stalk cells are fated to remain within the stalk portion. For ret-expressing tip cells, GDNF plays an important role in their maintenance. Bone morphogenetic protein 4 and FGF1 or FGF10 have been suggested to facilitate the elongation of the stalk (2, 3); however, factors that largely or exclusively cause proliferation and epithelial differentiation without branching, such as occurs in elongating stalks, have not been reported.

For an isolated UB to grow and branch robustly *in vitro*, GDNF alone is not sufficient. Conditioned medium from metanephric mesenchyme derived cells (BSN-CM) is required. Previously, we isolated PTN from this conditioned medium and were able to demonstrate that a combination of PTN and GDNF is sufficient to induce *in vitro* branching morphogenesis of the isolated UB (4). During the fractionation of this medium and isolation of PTN, the presence of a potent non-branch-promoting yet growth-inducing factor was noted.

Here, we have purified this factor from BSN-CM and have identified it as heregulin (HRG). Close examination of the HRG-induced UB revealed that the cells of these non-branching UBs displayed a predominant stalk cell-like morphology and expressed markers of maturation, raising the possibility that HRG may act as a stalk-promoting factor.

MATERIALS AND METHODS

Growth Factors

Recombinant growth factors, GDNF, and HRGα were from R&D systems. FGF1 was purchased from Calbiochem.

Cell Culture

The metanephric mesenchyme-derived cell line (BSN cells) was cultured in Dulbecco's modified Eagle's medium/F12 supplemented with 10% fetal calf serum at 37 °C in an atmosphere of 5% CO_2. Conditioned medium was collected after incubation with serum-free Dulbecco's modified Eagle's medium/F12 for 3–4 days.

Protein Purification

2–3 liters of BSN-CM was concentrated ~50-fold by Ultrasette tangential flow devices (5K molecular weight cutoff; Gelman Sciences). Morphogenetic activity of BSN-CM was retained in the >5,000 Dalton fraction, but not in the <5,000 Dalton flow-through. The buffer of the

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[2] Recipient of an American Heart Association Scientist Development Award.
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[5] The abbreviations used are: GDNF, glial cell line-derived neurotrophic growth factor; PTN, pleiotrophin; FGF, fibroblast growth factor; UB, ureteric bud; HRG, heregulin.

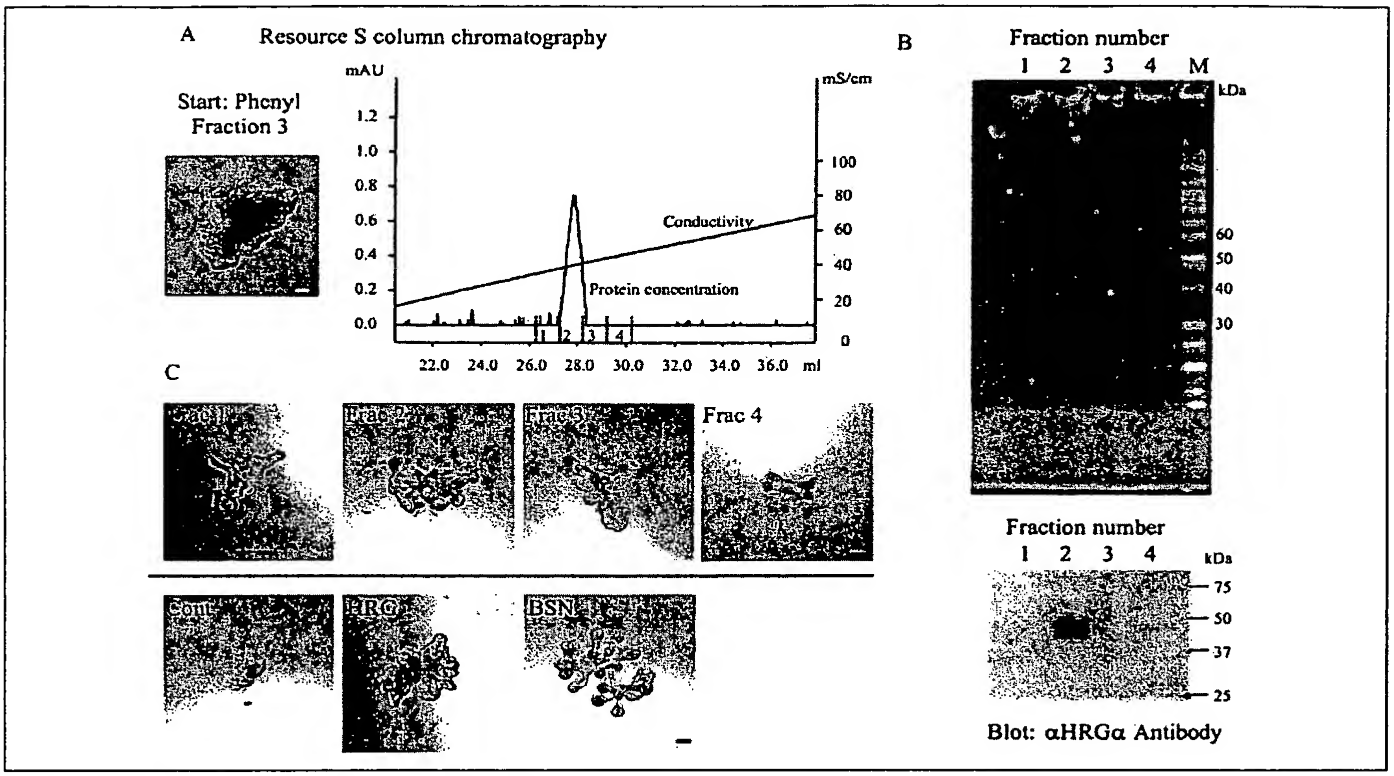


FIGURE 1. **Identification of non-branching growth inducing activity.** *A*, active fractions from a Resource Phenyl hydrophobic interaction column (*Fraction 3*) were applied to a Resource S cation exchange column. *Numbers* (*1–4*) in the chromatogram indicate fraction number. *B*, *fractions 1–4* were subjected to SDS-PAGE, followed by Sypro-Ruby staining (*upper panel*). *Fraction 2* has a band between 40 and 50 kDa, which was subsequently identified as HRGα by mass spectrometry-based method. Western blotting of these fractions probed with anti-HRGα antibodies confirmed the identification. *C*, morphogenetic activity of these fractions and indicated purified proteins was assayed by isolated rat UB culture. Photographs were taken at 5–6 days of culture. *Bars* = 100 μm.

concentrated BSN-CM was changed to 0.4 M NaCl, 50 mM Hepes, pH 7.2, buffer and applied to a heparin-Sepharose column (HiTrap Heparin 5 ml, Amersham Bioscience). Heparin-bound proteins were eluted via increasing concentrations of NaCl (0.4-2 M), and individual 5-ml fractions were collected. The 0.9–1.2 M NaCl heparin eluate fractions were adjusted to 1.7 M ammonium sulfate in 50 mM Hepes, pH 7.2, buffer and applied to a Resource Phenyl hydrophobic interaction column (Amersham Bioscience). Bound proteins were eluted via decreasing concentrations of ammonium sulfate (1.7-0 M) in 50 mM Hepes, pH 7.2, buffer. The 1.5–1.6 M ammonium sulfate fractions were dialyzed against 50 mM Hepes, pH 7.2, buffer and applied to a Resource S cation exchange column (Amersham Bioscience), followed by elution with increasing concentrations of NaCl (0–1 M). The morphogenetic activity of each fraction collected from this column was assayed using the isolated rat UB culture system (see below). An apparently homogenous protein band (see Fig. 1*B*, *lane 2*) was excised from Sypro-Ruby (Bio-Rad) stained Tris-glycine SDS-polyacrylamide gels (Cambrex) and analyzed by in gel digestion followed by microcapillary HPLC and tandem mass spectrometry (Harvard Microchem).

In Vitro Morphogenesis Assay

Isolated Rat UB Culture—As previously described (4, 5), UBs were microdissected from E13 rat embryonic kidney and suspended within extracellular matrix gels (1:1 mixture of type 1 collagen and growth factor reduced Matrigel, BD Biosciences), applied to the inner wells of a 24-well Transwell insert (Costar, 0.4-μm pore size). UBs were cultured in either whole BSN-CM (positive control), Dulbecco's modified Eagle's medium/F12 (negative control), dialyzed fractions (to Dulbecco's modified Eagle's medium/F12) or recombinant HRG (250–500 ng/ml); 125 ng/ml of GDNF, 250 ng/ml of FGF1, and 10% fetal calf serum, were also added to the outer wells in most cases. These cultures were placed in a 37 °C, 5% CO_2 incubator. Phase-contrast pictures were taken with Spot RT digital camera attached to Nikon microscope. In some cases, cultures were stained with rhodamine-conjugated phalloidin (Molecular Probes) and observed by scanning laser confocal microscopy (Zeiss LSM-510). Image analysis was done by Image Pro software.

Electron Microscopy—Isolated UBs cultured for 5 days in the presence of HRG as described above were fixed in Karnovsky's fixative for 1 h at room temperature and processed for ultrastructural analysis as described previously (3). Thin sections were mounted onto mesh grids, stained with lead citrate and uranyl acetate, and examined with a Zeiss EM109 microscope.

BrdUrd Incorporation Assay—As previously described (3), cultured ureteric buds grown for 4–5 days were labeled with 10 μM BrdUrd for 30 min. After extensive washing with phosphate-buffered saline, the UBs were fixed in 4% paraformaldehyde in phosphate-buffered saline. The incorporated BrdUrd was detected following a protocol of the Cell Proliferation kit (Amersham Biosciences) except for the use of fluorescein-conjugated anti-mouse IgG (Jackson ImmunoResearch) as a secondary antibody. After counterstaining with rhodamine-conjugated *Dolichos biflorus* lectin (Vector Lab), the samples were analyzed by scanning laser confocal microscopy (Zeiss LSM-510).

cDNA Array—For each treatment (HRG, BSN-CM, PTN), three isolated UBs were cultured in the presence of FGF1, GDNF, and indicated

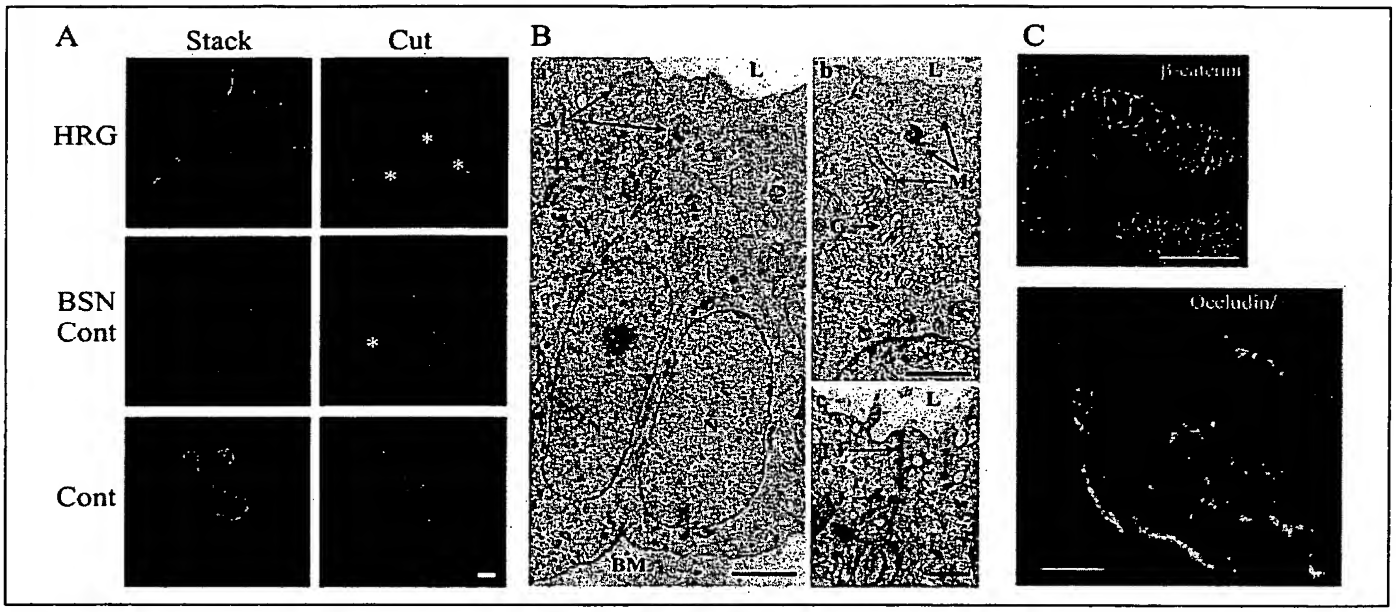


FIGURE 2. *A*, confocal analysis of isolated rat UBs grown in the presence of FGF1 (250 ng/ml) and GDNF (125 ng/ml) supplemented with 500 ng/ml of HRGα (*HRG*) or BSN-CM (*BSN Cont*), or without any supplementation (*Cont*) for 12 days. Buds were fixed and stained with rhodamine-conjugated phalloidin. *Left panels* are three-dimensional reconstructed confocal images, and *right panels* are optical sections at the middle of these structures. Note that HRG-treated UBs developed non-branching structures with large lumens. Lumens are indicated by *. *Bars* = 50 μm. *B*, electron microscopic analysis of UBs cultured for 5 days in the presence of 500 ng/ml HRG. *a*, composite photograph of polarized epithelial cells representative of the cells comprising HRG-treated UBs. Cells exhibit a typical polarized epithelial phenotype with nuclei (*N*) localized to the base of the columnar cells. Apical aspects of the cells abut a clear lumen (*L*). *Bar* = 3 μm. *b*, higher magnification examination of the apical portion of such cells. The apical ends of the cells appear to be highly secretory with clear Golgi bodies (*G*) and an abundance of rough endoplasmic reticulum. *Bar* = 1.5 μm. *c*, higher magnification of the cells showing the junctional apparatus. Here tight junctions (*TJ*) and adherens junctions (*AJ*) are clearly visible. Numerous secretory vesicles (*arrowheads*), some of which are associated with the apical plasma membrane, are also visible. *Bar* = 0.5 μm. *M*, mitochondria; *BM*, basement membrane. *C*, whole mount immunohistochemistry of UBs cultured for 5 days in the presence of HRG (500 ng/ml). The *upper panel* shows positive peri-junctional staining of β-catenin (*red*), and the *lower panel* shows positive apical staining of occludin (*green*) and Topro-3 nuclear staining (*blue*). *Bar* = 50 μm.

factor for 4 days. For each condition, 3 separate wells were prepared. RNA was isolated using PicoPure RNA isolation kit (Arcturus). Double IVT reaction and hybridization to Affymetrix rat gene chip 230A was performed at UCSD Cancer center GeneChip core facility. GCOS (Affymetrix) and Genesifter (VizX lab) software was used for data analysis. The gene expression data are available in the Gene Expression Omnibus website (ncbi.nlm.nih.gov/projects/geo/, accession number GSE3394).

Immunohistochemistry—Whole-mount staining of isolated UBs in three-dimensional culture was performed as described elsewhere (6). Antibodies/staining reagents were as follows: Topro-3 nuclear staining dye (1:1000, Molecular Probes), goat anti-rat GFRα1 antibody (1 μg/ml, R&D systems), rabbit anti-occludin antibody (1:100, Zymed Laboratories Inc.), and mouse anti-β-catenin monoclonal antibody (1:200, BD Transduction Lab). The samples were observed with Zeiss LSM-510 confocal microscopy.

RESULTS

BSN-CM Contains Non-branching Growth-inducing Activity for the Isolated UB—Fractionation of BSN-CM by multiple sequential liquid column chromatography has consistently revealed the presence of several growth-inducing activities in BSN-CM including a non-branching yet growth-inducing activity as well as a branch-promoting activity and an inhibitory activity. Thus far, a branch-promoting factor (PTN) (4) and growth/branch-inhibiting factors (transforming growth factor-β superfamily) (7) have been identified. In this study, we have purified a non-branching growth-inducing factor, which on a heparin-Sepharose column was found to elute prior to the branch-promoting activity (data not shown). This fraction was further separated on a Resource Phenyl-Sepharose column, and the non-branching growth-inducing activity was found to elute around 1.5–1.6 M ammonium sulfate (Fig. 1*A*).

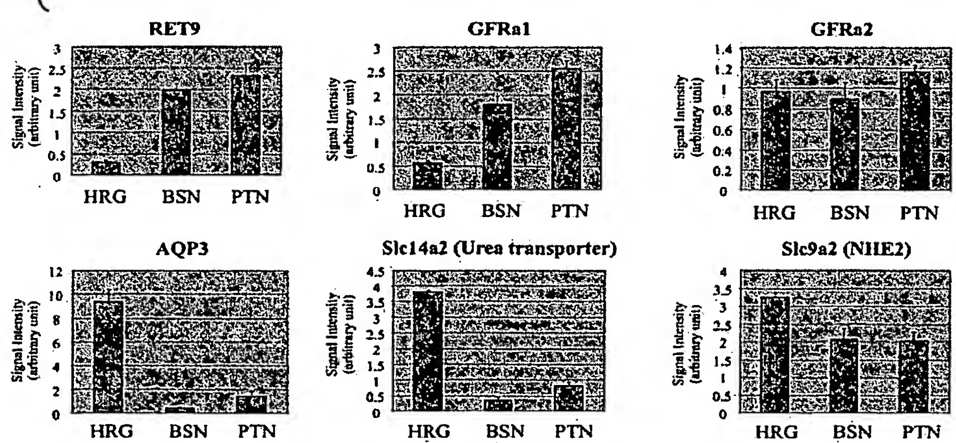
Heregulin α in BSN-CM Induces Non-branching Growth of the Isolated UB—This fraction from the Resource Phenyl column was further separated by Resource S cation exchange chromatography, and an apparently homogenous protein containing fraction with non-branching growth inducing activity was obtained (Fig. 1). The homogenous protein band eluted from the Resource S column at 0.6 M NaCl (in Fig. 1*B*, a band between 40 and 50 kilodalton present in *fraction 2*) was subsequently identified as HRGα by mass spectrometry. As expected, recombinant human HRGα induced non-branching growth of the isolated UB comparable with that seen with the native purified protein (Fig. 1*C*).

Heregulin-α Induces Non-branching Polarized Epithelial Proliferation of the Ureteric Bud in Three-dimensional Culture—To further evaluate the morphogenetic effect of HRGα, isolated UBs were cultured in the presence and absence of HRGα for several days. HRG stimulated overall growth of the isolated UB in the presence of GDNF and FGF1, compared with growth in the presence of only GDNF and FGF1, leading to the formation of non-branching structures morphologically distinct from BSN-CM-treated UBs (Fig. 1*C*). Confocal microscopic examination of phalloidin-stained UBs revealed the presence of a clear lumen with a much larger caliber in HRG-treated UBs compared with controls (Fig. 2*A*). As shown in Fig. 2*B*, electron microscopy demonstrated that the cells comprising these structures were epithelial in nature with characteristics usually associated with polarized epithelial cells. For example, the nuclei were uniformly localized to the basal portion of the cells, and intercellular junctions were readily apparent, presumably leading to the separation of basolateral and apical membrane domains. The apical

A

Comparison	Ontology	List	Up	Down	Array	z-up	z-down
HRG vs PTN	physiological process	363	156	207	3257	0.14	2.26
	metabolism	257	121	136	2007	3.81	2.17
	cellular process	238	114	124	2444	-0.39	-3.1
	protein metabolism	95	56	39	821	3.11	-1.67
	cell communication	92	43	49	1201	-2.32	-3.34
	development	83	51	32	770	2.7	-2.38
	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	78	47	31	623	3.55	-1.15
	transport	73	19	54	907	-4.32	-0.04
	signal transduction	67	28	39	888	-2.57	-2.27
	cell proliferation	61	47	14	344	8.11	-1.56
	morphogenesis	60	37	23	543	2.41	-1.85
	organogenesis	57	35	22	512	2.36	-1.72
	protein modification	56	37	19	406	4.34	-1.17
	cell cycle	44	37	7	237	8.08	-2.03
	phosphate metabolism	40	23	17	308	2.32	-0.35
	phosphorus metabolism	40	23	17	308	2.32	-0.35
	carboxylic acid metabolism	34	9	25	183	0.1	4.49
	organic acid metabolism	34	9	25	183	0.1	4.49
	cell surface receptor linked signal transduction	33	17	16	516	-1.69	-2.96
	phosphorylation	33	20	13	260	2.29	-0.69
HRG vs BSN	physiological process	312	167	145	3257	-0.18	2.35
	cellular process	211	130	81	2444	0.6	-3.31
	metabolism	208	103	105	2007	-0.07	3.59
	cell communication	90	58	32	1201	-0.61	-3.06
	development	77	61	16	770	3.88	-3.19
	transport	73	35	38	907	-2.02	0.11
	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	72	49	23	623	3.34	-0.59
	signal transduction	62	41	21	888	-0.82	-3
	morphogenesis	51	41	10	543	2.72	-2.88
	organogenesis	49	39	10	512	2.7	-2.65
	cell proliferation	46	34	12	344	4.15	-0.62
	cell cycle	38	29	9	237	5.09	-0.26
	cell surface receptor linked signal transduction	37	26	11	516	-0.13	-2.44
	lipid metabolism	33	5	28	248	-2.31	5.87

B



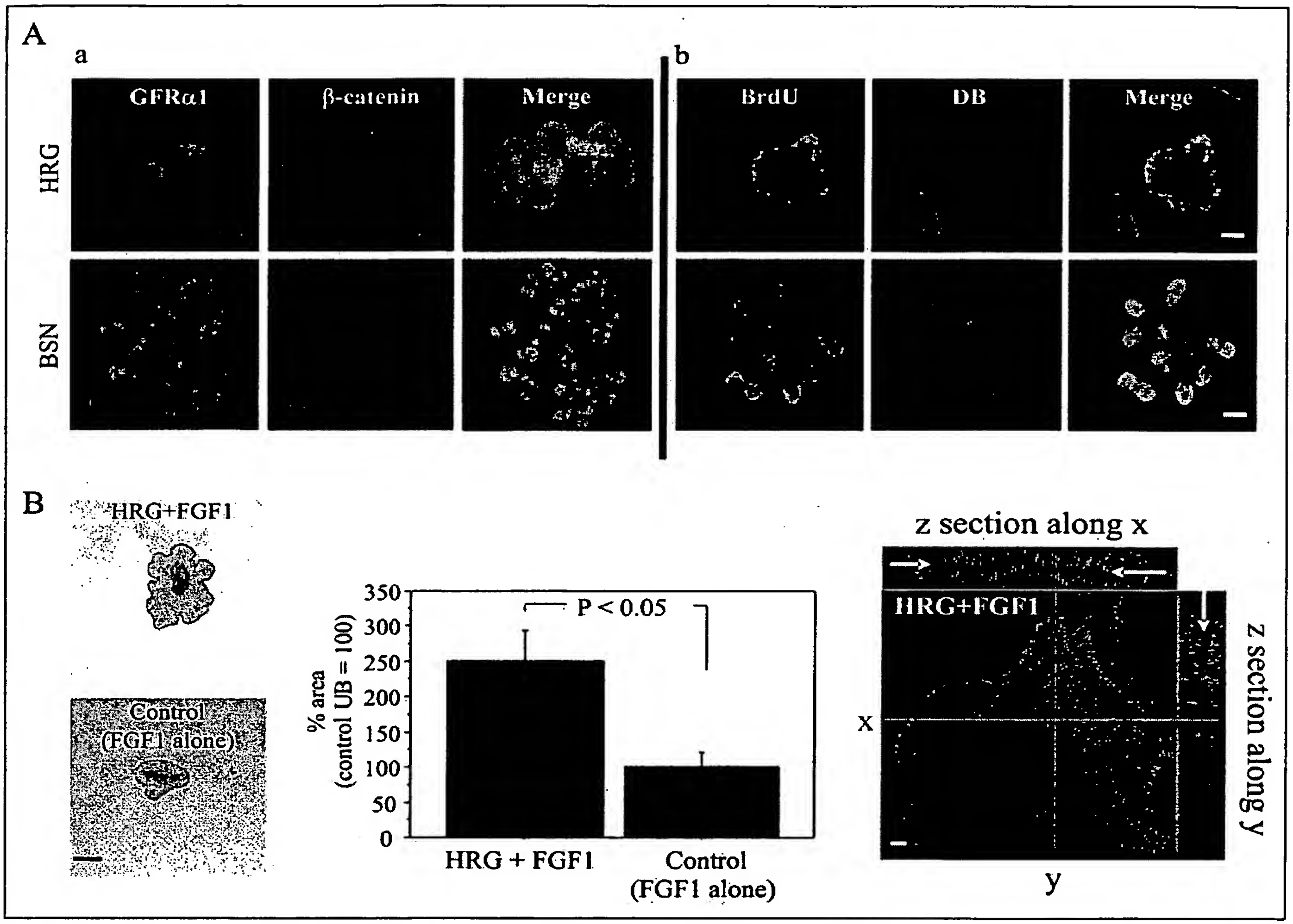


FIGURE 4. *A*, whole mount immunostaining of isolated UB cultured in the presence of HRG or BSN-CM plus FGF1 and GDNF. *a*, GFRα1 (*green*) and β-catenin (*red*) staining of isolated UB grown in the presence of HRG or BSN-CM and FGF-1 and GDNF. *b*, BrdUrd (*BrdU*) incorporation in isolated UBs in three-dimensional culture. Rat isolated UBs were cultured in the presence of either HRG or BSN-CM plus FGF1 and GDNF for 5 days at which time the UBs were labeled with BrdUrd, which was detected with mouse anti-BrdUrd antibodies and fluorescein isothiocyanate-conjugated anti-mouse IgG (*green*). UBs were counterstained with rhodamine-conjugated lectin *D. biflorus* (*DB*) (*red*). Pictures presented here are three-dimensional reconstruction images from confocal microscopy. *Bar* = 100 μm. *B, left*, phase contrast pictures of rat isolated UBs cultured in the presence of HRG (500 ng/ml) and FGF1 (250 ng/ml) or FGF1 alone (control) for 1 week. Note that UBs in these experiments were cultured in the absence of GDNF. *Bar* = 100 μm. The graph shows quantification of UB area between HRG-treated UBs and control UBs. *Error bars* indicate S.E. $n = 5$ for each condition. *Right*, confocal microscopic analysis of HRG-treated UBs in the absence of GDNF stained with β-catenin. *Arrows* indicate lumens. *Bar* = 20 μm.

aspect of the cells contained multiple secretory granules, which can be seen in contact with the luminal surface (Fig. 2*B*, *c*, *arrows*). As expected, the adherens junction-associated protein, β-catenin, was localized at the site of cell-cell contact, and the tight junction protein, occludin, was localized at the luminal side (Fig. 2*C*).

Heregulin Treatment Does Not Maintain GFRα1 Expression and Leads to GDNF-independent Growth and Maturation of the Ureteric Bud—We next sought to investigate how HRG induced non-branching growth. To address this question, the gene expression profile of either whole BSN-CM or PTN-treated branching UBs was compared with the HRG treated UBs on cDNA arrays. First, the overall gene expression profile among HRG-treated, non-branching UBs, and either BSN-CM- or PTN-treated branching UBs was compared using the MAPPFinder algorithm built into the Genesifter application) (8). This analysis provides information on the probability (*i.e.* Z score) that a change in expression of a group of genes (*i.e.* ontological class) is because of random chance or is likely to be a real effect. A gene was included in the analysis when the gene was determined to be either present in all three

FIGURE 3. **Differential mRNA expression profile of non-branching UBs (HRG-treated) and branching UBs (BSN-CM or PTN-treated) obtained from cDNA array analysis.** *A*, differentially expressed genes (expression level > 1.5-fold at $p < 0.05$) were categorized according to their ontology as obtained by Genesifter software. HRG-treated condition was compared with either PTN-treated (*upper*) or BSN-CM treated (*lower*) conditions. Numbers in the *List* column indicate total number of differentially regulated genes. Ontological classes containing more than 32 differentially expressed genes were listed. *Up/Down* columns indicate the numbers of genes up-regulated or down-regulated in branching (PTN or BSN-CM treated) conditions in comparison with non-branching (HRG-treated) condition. Numbers in the *Array* column indicate total number of genes in each ontology class on cDNA array. Extreme numbers (>2 or < −2) in the Z score columns indicate that a certain process is either over-represented or under-represented among the differentially expressed genes. *B*, expression levels of GDNF receptor genes (*Ret* and *Gfra1*) and those encoding transporters expected to be present in the mature collecting duct (aquaporin3 (*AQP3*), urea transporter, and sodium-hydrogen exchanger2 (*NHE2*)) were compared among branching and non-branching UBs. Note that another *Gfra* family gene, *Gfra2* was not differentially expressed among these conditions. Expression level was normalized to median of all experiments. *Error bars* indicate S.E.

experiments or present in two experiments and one marginal in at least one treatment condition. Of these genes, the differentially regulated (*i.e.* >1.5-fold at $p < 0.05$) genes were categorized according to their ontology (Fig. 3*A*). Genes up-regulated by PTN treatment were over-represented (Z score > 2) by genes thought to be involved in cell proliferation and development, whereas transport-related genes were under-represented (Z score < −2). Genes thought to be involved in acid metabolism were over-represented in the group of genes down-regulated by PTN. Genes involved in cell communication, signal transduction, and developmental processes were under-represented in groups down-regulated by PTN. Similar to the comparison of HRG *versus* PTN, genes involved in cell proliferation and development were over-represented among those genes up-regulated by BSN-CM treatment. Genes involved in metabolism were over-represented among those genes down-regulated by BSN-CM treatment, whereas genes involved in cell communication, signal transduction, and development were under-represented in this group of genes. From this data analysis, we hypothesized that HRG-treatment decreases the overall proliferation of isolated UBs compared with pro-branching factors such as BSN-CM and PTN and directs them toward maturation. When we examined specific gene expression, it was noted that the expression level of both the binding receptor for GDNF, GFRα1, and the signal transducing receptor, ret, were down-regulated in the HRG-treated condition (Fig. 3*B*). On the other hand, consistent with the ultrastructural finding that mature appearing epithelial cells were lining HRG-treated UBs, the expression level of transporter genes expressed in the mature collecting duct, such as aquaporin3, the urea transporter, and sodium hydrogen exchanger 2, was up-regulated in HRG-treated condition (Fig. 3*B*).

The down-regulation of GFRα1 was confirmed at the protein level by immunostaining (Fig. 4*A*, *a*). In the BSN-CM-treated branching UB, GFRα1 was localized predominantly at the UB tip, whereas its expression was dramatically reduced in the HRG-treated UB. This expression pattern of GFRα1 raised the possibility that the "tip cell phenotype" is diminished in the HRG-treated UB. Because most of the proliferating cells found in the UB have been shown to localize at the tip (1, 3, 6, 9), we examined the pattern of proliferation by BrdUrd incorporation. In contrast to the BSN-CM-treated branching UB, in which most proliferating cells are localized at the tip, proliferating cells in HRG-treated UB displayed no specific localization (Fig. 4*A*, *b*). Together with down-regulation of tip-localized GDNF receptors, *Gfra1* and *Ret*, HRG is likely to stimulate non-GDNF-dependent UB cell proliferation that may be operational in stalk growth. The fact that HRG-treated UBs did not grow as large as BSN-CM-treated UBs (Fig. 4*A*), together with overall down-regulation of cell proliferation genes in the HRG-treated condition *versus* BSN-CM-treated condition, suggests that this proproliferative effect is weaker than that of tip proliferation-inducing factors.

Nevertheless, we further went on to confirm the ability of HRG to induce UB cell proliferation independent of GDNF. It was possible to maintain isolated UB survival in the presence of FGF1 without significant growth (Fig. 4*B*, control). Addition of HRG (without GDNF present) to this condition facilitated the growth as indicated by the increase in UB area (Fig. 4*B*). These HRG-treated UBs appeared to retain tubular epithelial structure with lumen as shown by confocal microscopy (Fig. 4*B*).

DISCUSSION

Taken together, our data suggest that HRG does not maintain the tip cell phenotype but promotes differentiation of the isolated UB, making it all stalk phenotype. Moreover, non-branching growth of the UBs induced by HRG treatment is independent of GDNF, raising the possibility that tip and stalk cells in the branching epithelial tissue are under the control of different sets of growth factors.

BSN-CM, derived from metanephric mesenchyme cells, contains at least three apparently independent morphogenetic activities that stimulate the isolated UB in three-dimensional culture: 1) a branch-promoting activity, 2) a non-branching growth-inducing activity, and 3) an inhibitory activity (4, 7). The presence of these multiple activities is consistent with a model of UB branching where multiple soluble factors secreted from the metanephric mesenchyme, perhaps under different spatial and temporal conditions, regulate the growth and shape of the UB-derived tubular tree. This is very different from cell culture models of branching in which a single growth factor (*e.g.* hepatocyte growth factor in MDCK cells or epidermal growth factor receptor ligands in mIMCD3 cells) is sufficient to induce growth and branching. Previous studies have identified proteins likely to be responsible for the branch-promoting (3–5) and inhibitory activities (7) present in BSN-CM. In this study, we have isolated a non-branching yet growth-inducing factor from BSN-CM and have identified it as HRGα. Both natural and recombinant HRG reproducibly induced the non-branching growth of the isolated UB in three-dimensional culture.

HRGα was originally isolated from a breast cancer cell line as an activator of the oncogene/transmembrane tyrosine kinase receptor erbB2 (10, 11). HRGα is 1 of at least 15 isoforms differentially spliced from the neuregulin1 transcript (12). These neuregulin1 isoforms signal through their receptors erbB2, -3, and -4 (13) and have been shown to be critical in development of the central and peripheral nervous system as well as the heart (14–16) and mammary gland (17). Neuregulin1 transcripts have been detected in mesenchyme of the lung, intestine, stomach, kidney, and the genital ridge in early mouse development (18). In organ culture of the mammary gland, HRG has been shown to stimulate alveolar differentiation, whereas different growth factors stimulate branching morphogenesis (19, 20).

Our results using the isolated UB culture system are potentially compatible with this observation. HRG did not stimulate branching morphogenesis, but it promoted growth and maturation of the UB. Cells comprising these structures were epithelial in nature with characteristics associated with tight polarized epithelial cells (Fig. 2). We demonstrated that HRG-induced growth could be explained by loss of total and differential expression of GFRα1 (Figs. 3 and 4). We speculate that the presence of GFRα1 in cells at the branching tip might be a prerequisite for the UB to undergo branching morphogenesis. Thus, if GFRα1 were ubiquitously expressed along the UB at a relatively high level, the UB would be expected to display an "all-tip" phenotype, resulting in globular growth without any apparent stalk formation. On the other hand, if GFRα1 expression were reduced or non-existent, tip cells would not be able to respond to GDNF and would be expected to display an "all-stalk" phenotype. We suggest this is the case in UBs treated with HRG. Moreover, losing tip phenotype from the UB seems to confer maturation toward the collecting duct epithelium. Other conditions that lead to down-regulation of ret and GFRα1 (*e.g.* FGF1 alone or FGF1 and GDNF) also induced increased transporter/water channel expression as seen with HRG treatment.[6]

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